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Anti-tumor immunity induced by full-length human ERBB-2 DNA constructs was ranked as wild-type ERBB-2 (E2) > tyrosine kinase deficient ERBB-2 (E2A) > cytoplasmic E2 (cytE2) > cytE2A. Cytoplasmic E2 and its tyrosine kinase deficient counterpart cytE2A, both encode a protein targeted to and rapidly degraded in the cytosol by the proteasomes. Anti-ErbB-2 antibody was induced by immunization with transmembrane, but not cytoplasmic, ERBB-2 DNA. Although an excellent vaccine against D2F2/E2, vaccination with E2 DNA was only moderately effective against D2F2/cytE2 tumor. Protection against both D2F2/E2 and D2F2/cytE2 was achieved by co-vaccination with cytE2 or cytE2A and GM-CSF or IL-2 DNA, indicating effective immunogenic peptide presentation from cytoplasmic ErbB-2 and a need for costimulation. ErbB-2 specific CTL were detected in mice immunized with cytE2A and GM-CSF after tumor rejection. Co-vaccination with E2A and cytE2A induced synergistic anti-tumor activity, supporting enhanced peptide presentation from cytoplasmic ErbB-2. Transmembrane ErbB-2 DNA vaccination elicited both humoral and cellular immune responses and protected mice from D2F2/E2. Cytoplasmic ERBB-2 and cytokine DNA vaccination induced anti-tumor cellular, but not humoral responses. This study demonstrates the feasibility of eliciting individual effector mechanisms by vaccination with targeted DNA constructs and protection against ErbB-2 expressing tumors without antibody activity.

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Table of Contents

Front Cover ---------- 1

Report Documentation Page ---------- 2

Table of Contents ---------- 3

Introduction ---------- 4

Body of Proposal ---------- 4

Key Research Accomplishments ---------- 6

Reportable Outcomes ---------- 6

Conclusions ---------- 6

Appendices ---------- 7

- Acronym and Symbol Definition ---------- 7
- Publications ---------- 8

INTRODUCTION

The goal of this study is to prevent the growth or recurrence of breast cancer by active vaccination with a tumor antigen, ErbB-2. The specific objectives are to generate and test recombinant DNA vaccines which can induce a strong anti-tumor immune response. Human tumor associated antigens, such as ErbB-2, are generally self antigens and may be associated with transforming activities. In our recombinant vaccines, the transforming activity of ERBB-2 is eliminated by point mutation. Recombinant ErbB-2 molecules are directed to the subcellular compartments of antigen processing and presentation and the generation of an anti-tumor immune response is characterized. Co-vaccination with cytokine genes such as IL-2 or GM-CSF are also tested. The reagents developed in this study will be candidate breast cancer vaccines and the principles established by this study will be applicable to new tumor antigens.

SPECIFIC TASKS

1 Continue to modify and test recombinant cytoplasmic erbB-2 which are free of tyrosine kinase activity
2 Construct and test recombinant ERBB-2 which is targeted to MHC II antigen processing pathway
3 Enhance vaccine efficacy by local IL-2 secretion and by expression with adenoviral vectors

STUDIES AND RESULTS

Task 1 **Continue to modify and test recombinant cytoplasmic ErbB-2 which are free of tyrosine kinase activity.**

As described in the last report, several recombinant ERBB-2 DNA constructs have been generated in our lab. Of these, ERBB-2A (E2A) and cytoplasmic ERBB-2A (cytE2A) contained a lysine to alanine point mutation at amino acid 753 to eliminate ATP binding and were not phosphorylated. The cytoplasmic ErbB-2 constructs, lacking the ER signal sequence, are directed to the cytoplasm and promptly degraded by the proteosomes. Cytoplasmic ErbB-2 protein could be detected only after transfected cells were incubated overnight with a proteasome inhibitor. The immunogenicity and vaccination efficacy of cytoplasmic ERBB-2 DNA was further studied and is reported in task 3.

Task 2 **Construct and test recombinant ERBB-2 which is targeted to MHC II antigen processing pathway**

To activate CD4 T cells, a recombinant ERBB-2 (E2-Lamp) was generated in our lab. E2-Lamp encodes a fusion protein with the extracellular and transmembrane domains of ERBB-2 fused to the cytoplasmic tail of the lysosomal-associated membrane protein (lamp-1). This construct is expected to

direct the recombinant protein to the lysosome where it can be degraded for presentation with MHC class II molecules. I will test the immunogenicity and vaccination efficacy of E2-Lamp and if necessary, make further modifications to enhance anti-ErbB-2 CD4 T cell activation.

Task 3 **Enhance vaccine efficacy by local IL-2 secretion and by expression with adenoviral vectors**

3.1 Anti-tumor immunity induced by recombinant ERBB-2 constructs

A manuscript has been submitted for publication and is attached. Vaccination with E2, E2A, cytE2, or cytE2A resulted in approximately 90, 60, 30 and 10% protection against D2F2/E2 tumor, respectively. All recombinant proteins contained the entire ErbB-2 structural sequence, but the subcellular localization, membrane stability, and tyrosine kinase activity significantly affected their immunogenicity. E2A is a tyrosine kinase deficient ErbB-2 mutant containing a single amino acid substitution. Cytoplasmic E2 and its tyrosine kinase deficient counterpart cytE2A, both encode a protein targeted to and rapidly degraded in the cytosol by the proteasomes. Anti-ErbB-2 antibody was induced by immunization with transmembrane, but not cytoplasmic, ERBB-2 DNA. The same pattern, but elevated antibody response was observed in mice bearing mouse mammary tumor D2F2 expressing the corresponding ErbB-2 derivative. Vaccination with transmembrane ERBB-2 induced IgG2a production which is indicative of a Th1 response and CD4 T cells were required for antibody production. Although an excellent vaccine against D2F2 expressing wild-type ErbB-2 (D2F2/E2), vaccination with E2 DNA was only moderately effective against D2F2 tumor expressing cytoplasmic ErbB-2 (D2F2/cytE2). Because challenge with D2F2/E2 enhanced anti-ErbB-2 antibody production by E2 vaccination, antibody activity may have contributed to tumor rejection. Anti-ErbB-2 antibody would not affect D2F2/cytE2 tumor growth and the tumor itself does not enhance antibody production. Protection against both D2F2/E2 and D2F2/cytE2 was achieved by co-vaccination with cytE2 or cytE2A and GM-CSF or IL-2 DNA, indicating effective immunogenic peptide presentation from cytoplasmic ErbB-2 and a need for costimulation. ErbB-2 specific CTL were detected in mice immunized with cytE2A and GM-CSF after rejection of either D2F2/E2 or D2F2/cytE2. A standard chromium release assay was performed using D2F2 or D2F2/E2 as target cells. Specific lysis of D2F2/E2 correlated with CTL from mice which had rejected tumor. No specific lysis was detected in any tumor bearing mouse regardless of vaccination. No lysis of the D2F2 parent tumor was detected. Co-vaccination with E2A and cytE2A induced synergistic anti-tumor activity, supporting enhanced peptide presentation from cytoplasmic ErbB-2. Therefore, transmembrane ERBB-2 DNA vaccination elicited both humoral and cellular immune responses and protected mice from D2F2/E2. Cytoplasmic ERBB-2 and cytokine DNA vaccination induced anti-tumor cellular, but not humoral response. This study demonstrates the feasibility of eliciting individual effector mechanisms by vaccination with targeted DNA constructs and protection against ErbB-2 expressing tumors without antibody activity.

3.2 **Generation of adenoviral vector with cytE2 and verification of protein expression.**

Adenovirus will not be constructed as the infectious properties of adenovirus and the oncogenic potential of ErbB-2 may create greater risk than benefit. Instead, E2-LAMP or cytE2 will be modified to contain a fragment from a virus. This construct will contain foreign peptides which may enhance T cell responses and overcome potential tolerance.

KEY RESEARCH ACCOMPLISHMENTS

- Anti-ErbB-2 antibody of the IgG2a subset was inducted by vaccination with transmembrane but not cytoplasmic ERBB-2
- Anti-tumor immunity against both D2F2/E2 and D2F2/cytE2 was induced when mice were vaccinated with cytE2A and IL-2 or GM-CSF genes in the absence of antibody production.
- Vaccination with cytE2A and GM-CSF was superior to E2 vaccination in protection against tumor expressing cytoplasmic ErbB-2.
- Anti-ErbB-2 CTL was detected only in mice which had rejected tumor.

REPORTABLE OUTCOMES

Publication 3 & 4 are attached

1. Wei, W. Z., Shi, W. P., Galy, A., Lichlyter, D., Hernandez, S., Groner, B., Heilbrun, L.,and Jones, R. F.; (1999): Protection against mammary tumor growth by vaccination with full-length, modified human ErbB-2 DNA. *Int.J.Cancer* 81, *1-7.*

2. Pilon, S., Kelly, C., Marriott, E., and Wei, W.Z.; (1999): Protection against mammary tumor growth by vaccination with recombinant ERBB-2 DNA encoding transmembrane or cytoplasmic protein. *The Faseb Journal* 13, A645.

3. Pilon, S.A., Piechocki, M.P., and Wei, W.Z.; (2000): IL-2 or GM-CSF substitute for CD4 T cell help in cytERBB-2 DNA vaccination to induce anti-tumor immunity. *AACR Proceedings* 41, 5060.

4. Pilon, S.A., Piechocki, M.P., and Wei, W.Z.; Vaccination with cytoplasmic ErbB-2 DNA protects mice from mammary tumor growth without anti-ErbB-2 antibody. (submitted)

CONCLUSIONS

Native ErbB-2 is a transmembrane protein with tyrosine kinase activity. To induce ErbB-2 specific CTL, a recombinant cytoplasmic ErbB-2 which lacks kinase activity was constructed. This recombinant protein localizes in the

cytoplasm and is rapidly degraded by the proteosome. Vaccination with cytE2A alone does not protect against an ErbB-2 expressing tumor. This may be due to a lack of CD4 T cell induction. To activate or replace ErbB-2 specific CD4 T cells, co-vaccination of cytE2A with cytokine genes was examined. Co-vaccination of cytE2A with either IL-2 or GM-CSF genes induced an effective anti-tumor immune response and anti-ErbB-2 CTL. These results indicate the processing and presentation of ErbB-2 antigenic epitopes from cytoplasmic ErbB-2 and that IL-2 or GM-CSF can substitute CD4 co-stimulation generated from transmembrane ErbB-2.

Acronym and Symbol Definition

CTL	Cytotoxic T cell
cytE2A	ERBB-2 lacking ER signal sequence and lacking tyrosine kinase activity
E2A	ERBB-2 lacking tyrosine kinase activity
ER	Endoplasmic reticulum
ErbB-2	Transmembrane protein
ERBB-2	Gene encoding transmembrane protein ERBB-2
GM-CSF	Granulocyte-macrophage colony stimulating factor
IL-2	Interleukin 2
MHC	Major histocompatibility complex

Publications

AACR 2000

IL-2 OR GM-CSF SUBSTITUTE FOR CD4 T CELL HELP IN cytERBB-2 DNA VACCINATION TO INDUCE ANTI-TUMOR IMMUNITY.

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As we previously reported, vaccination with DNA encoding full-length transmembrane ERBB-2 (E2), but no cytoplasmic ERBB-2 (cytE2), protected BALB/C mice against D2F2 mammary tumor expressing E2. CytE2 and its tyrosine kinase deficient derivative (cytE2A) are targeted to the cytosol where rapid degradation by the proteosome should generate a complete repertoire of MHC class I associated E2 peptides. The absence of anti-tumor immunity following cytE2 DNA vaccination may be due to a deficient CD4 T cell or B cell response. Vaccination with transmembrane, but not cytoplasmic, E2 DNA induced an anti-ErbB-2 antibodies of the IgG2a subset, indicative of a Th1 response. Antibody production was eliminated when CD4 T cells were depleted. To test if co-vaccination with cytokine genes compensated for CD4 help, mice were immunized with pCMV/cytE2A and pEF-BOS/IL-2 or pEF-BOS/GM-CSF. Eighty percent of vaccinated mice rejected tumor, although anti-ErbB-2 antibodies were not detected. Therefore, at least a partial compensation of CD4 help was achieved with IL-2 or GM-CSF which resulted in tumor rejection. Vaccination with cytE2 DNA and challenge with a tumor expressing cytoplasmic E2 (D2F2/cytE2) resulted in enhance tumor growth. These results support T cell anergy or a suppressed anti-tumor immune response when MHC class I peptides are presented alone. Therefore, activation of a Th1 response and anti-ErbB-2 was indicative of, but not necessary for, anti-tumor immunity. Immunization with cytE2 alone induced T cell anergy or suppression but co-vaccination with cytokine genes compensated for the lack of CD4 help and induced anti-tumor immunity. DAMD17-98-1-8265 and CA76340.

Vaccination with cytoplasmic ErbB-2 DNA protects mice from mammary tumor growth without anti-ErbB-2 antibody[1]

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Abstract

Anti-tumor immunity induced by four full-length human ErbB-2 DNA constructs was ranked as native ErbB-2 (E2) > ErbB-2A (E2A) > cytoplasmic E2 (cytE2) > cytoplasmic E2A (cytE2A)[3]. E2A is a tyrosine kinase deficient ErbB-2 mutant containing a single amino acid substitution. Cytoplasmic E2 and its tyrosine kinase deficient counterpart cytE2A, both encode a protein targeted to and rapidly degraded in the cytosol by the proteasomes. Anti-ErbB-2 antibody was induced by immunization with transmembrane, but not cytoplasmic, ErbB-2 DNA. The same pattern, but elevated antibody response was observed in mice bearing mouse mammary tumor D2F2 expressing the corresponding ErbB-2 derivative. CD4 T cells were required for antibody production. Although an excellent vaccine against D2F2/E2, vaccination with E2 DNA was only moderately effective against D2F2/cytE2 tumor. Protection against both D2F2/E2 and D2F2/cytE2 was achieved by co-vaccination with cytE2 or cytE2A and GM-CSF or IL-2 DNA, indicating effective immunogenic peptide presentation from cytoplasmic ErbB-2 and a need for costimulation. ErbB-2 specific CTL were detected in mice immunized with cytE2A and GM-CSF after tumor rejection. Co-vaccination with E2A and cytE2A induced synergistic anti-tumor activity, supporting enhanced peptide presentation from cytoplasmic ErbB-2. Therefore, transmembrane ErbB-2 DNA vaccination elicited both humoral and cellular immune responses and protected mice from D2F2/E2. Cytoplasmic ErbB-2 and cytokine DNA vaccination induced anti-tumor cellular, but not humoral response. This study demonstrates the feasibility of eliciting individual effector mechanisms by vaccination with targeted DNA constructs and protection against ErbB-2 expressing tumors without antibody activity.

Introduction

ErbB-2 or Her-2/*neu*, a member of the epidermal growth factor receptor family, is overexpressed in several human cancers including breast, ovarian and lung cancers (1;2). The tyrosine kinase activity of ErbB-2 leads to cell proliferation and transformation. Overexpressed ErbB-2 is associated with aggressive disease and poor prognosis (3). With the detection of ErbB-2 specific antibody and T cells in breast and ovarian cancer patients, ErbB-2 is recognized as a target of immunotherapy (4-7). Various treatments directed at ErbB-2 including Herceptin, a humanized anti-ErbB-2 monoclonal antibody, have been administered to patients. Cardiac toxicity was exerted by Herceptin treatment particularly when the patients also received anthracyclines or cyclophosphamides (8). It is possible that cardiac toxicity mediated by the antibody may not be exerted by anti-ErbB-2 T cells. ErbB-2 vaccines that can induce tumor rejection without antibody may be particularly useful.

Although Herceptin demonstrated anti-tumor activity, the heterogenous antibodies induced by active vaccination may have negative effects on anti-tumor immunity. Some anti-ErbB-2 monoclonal antibodies have been shown to trigger positive signaling events resulting in enhanced tumor growth (9). Inhibition of anti-tumor T cell activity by antigen specific antibodies has been described (10;11). In mice transgenic for the rat ErbB-2 (neu) gene, neu specific antibodies generated by DNA vaccination were not required for tumor protection (12). On the other hand, inhibition of spontaneous tumorigenesis after rat neu DNA immunization was associated with anti-neu antibody, although T cell reactivity could not be ruled out (13). Anti-neu antibody induced by a neu expressing whole cell vaccine may contribute to tumor rejection in a different neu transgenic model although T cell activity was also required (14). Rhesus monkeys immunized with the extracellular domain (ECD) produced anti-ErbB-2 antibodies which inhibited the growth of breast cancer cell lines overexpressing ErbB-2 (15). The mixed activity of antibody and potential cardiac toxicity raises concerns about generating long-lasting, irreversible antibody production by vaccination.

The efficacy of anti-ErbB-2 T cells also needs clarification. CD8 and CD4 T cells were activated in patients immunized with HLA-A2.1 or HLA-DR associated ErbB-2 peptides. Peptide specific CTL, however, failed to lyse human cancer cells with amplified ErbB-2, leaving in question the efficacy of peptide immunization (16). Vaccination of rats with MHC class II associated peptides induced anti-neu antibody and T cell immunity but the anti-tumor efficacy was not clear (17). Continued clinical trials without insightful understanding of the various anti-ErbB-2 effector mechanisms and their relevance in tumor rejection may not be fruitful.

To delineate the effector components in anti-ErbB-2 immunity, a panel of human ErbB-2 DNA constructs were generated in our lab (18). E2A encodes full-length ErbB-2 with a single amino acid substitution to replace ATP binding lysine (K) with alanine (A) and to eliminate tyrosine kinase activity. CytE2 has a truncated endoplasmic reticulum (ER) signal sequence and encodes a protein that is released into the cytoplasm rather than transported into the ER as a transmembrane protein. CytE2A is cytE2 with the K to A mutation. Plasmid DNA

was chosen as the vaccine candidate because it is chemically defined, can be produced in large quantity, purified to homogeneity and is relatively stable. DNA can be readily modified to encode proteins with the desired biochemical, biological and thus immunological properties, making it possible to perform mechanistic analysis in a timely fashion. CytE2 and cytE2A are of particular interest because the proteins are targeted to the cytoplasm and rapidly degraded by the proteasome. Processing of proteins through this pathway should result in a complete repertoire of MHC I peptides for CD8 T cell recognition. Rapid degradation via proteasome is associated with enhanced peptide presentation and T cell reactivity (19). In our preliminary study, cytE2 or cytE2A vaccination was, however, poorly protective compared to the transmembrane counterparts. In this study, this observation was further analyzed and profound anti-tumor activity was achieved when co-stimulation signals were provided during cytE2 or cytE2A vaccination.

Materials and Methods

Animals and cell lines

BALB/c (6-8 wk old) mice were obtained from Charles River Laboratory (Frederick, MD) and The Jackson Laboratory (Bar Harbor, ME). D2F2 is a mouse mammary tumor line derived from a spontaneous mammary tumor that arose in a BALB/c hyperplastic alveolar nodule (HAN) line D2 (20). The human breast cancer cell line SKBR-3 which has amplified ErbB-2 was purchased from the ATCC (Manassas, VA). These cell lines were maintained *in vitro* in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated cosmic calf serum (Hyclone, Logan, UT), 10% NCTC 109 medium (Sigma, St. Louis, MO), 2 mM L-glutamate, 0.1 mM MEM non-essential amino acids, 100 units/ml penicillin, and 100μg/ml streptomycin. D2F2 lines co-transfected with recombinant ERBB-2 constructs and pRSV2/neo: D2F2/E2, D2F2/E2A, D2F2/cytE2, and D2F2/cytE2A, were maintained in medium containing 0.8 mg/ml G418 (Geneticin, Sigma). All tissue culture reagents were purchased from Gibco (Gaithersburg, MD) unless otherwise specified.

DNA Immunization

The recombinant ErbB-2 plasmids pCMV, pCMV/E2 (E2), pCMV/E2A (E2A), pCMV/cytE2 (cytE2), and pCMV/cytE2A (cytE2A) have been described previously (18). Plasmids, pEFBos/GM-CSF and pEFBos/IL-2, encoding murine GM-CSF and IL-2, were from N. Nishisaki (Osaka University, Osaka, Japan). BALB/C mice at 6-8 weeks of age received i.m. injections of plasmid DNA 1-2 μg/μl suspended in saline with 50 μl in each thigh. Vaccination was repeated 3 times at two week intervals.

Tumor Challenge

At two weeks after the final DNA vaccination, mice were challenged s.c. in the right flank with $2x10^5$ D2F2 tumor cells expressing wild-type or mutant ErbB-2. Tumors were measured weekly by a caliper in two dimensions, and mean tumor diameter was calculated. Animals were sacrificed when tumor diameter reached 10 mm.

Measurement of anti-ErbB-2 antibodies

Blood was collected from mice at one week after the third DNA vaccination or four weeks after tumor challenge. To measure anti-ErbB-2 antibody, SKBR3 cells were stained using mouse serum diluted 1:20 as the primary antibody. A fluorescein-conjugated goat anti-mouse γ-chain of pan IgG (Jackson ImmunoResearch, West Grove, PA), IgG1, or IgG2a (Caltag, Burlingame, CA) secondary antibody was used to detect bound serum IgG. The monoclonal antibody TA-1, which recognizes an extracellular domain of ErbB-2, was used as a positive control (Oncogene Research Products, Cambridge, MA). Normal mouse serum or isotype matched Mab was the negative control. Flow cytometric analysis was performed with a FACs Caliber (Becton Dickinson, San Jose, CA). Results are expressed as mean channel fluorescence.

CD4 T cell depletion

Monoclonal antibody, GK1.5 (ATCC) was used to deplete CD4 T cells. Each mouse was treated by i.p. injection of 500 μg of GK1.5 hybridoma ascites for three consecutive days and then every three days thereafter until the completion of the experiment. At six days after the first injection of GK1.5, animals were challenged s.c. with $2x10^5$ D2F2/E2 cells. Depletion was verified by FACs analysis of splenocytes six days after the first injection (data not shown).

Generation of CTL and CTL assay

Splenocytes from immunized mice were isolated at 6 weeks after tumor challenge by Ficoll separation and incubated with irradiated stimulator 3T3 cells transfected with wild-type ErbB-2 and K^d. Cultures were maintained in R10: RPMI 1640 supplemented with 10% FCS, 2mM L-glutamate, 50 μM 2-ME, 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco). On day 7, viable cells were analyzed for cytotoxic activity. D2F2 and D2F2/E2 cells were labeled with sodium [^{51}Cr] chromate for 2 hours at 37°C. In a 96-well round-bottom plate, target cells were incubated with responder cells at different E:T ratios for 4 hours at 37°C. Fifty microliters per well of supernatant was transferred to a 96-well plate with 100μl of Optiphase Supermix scintillation fluid and counted on a Trilux, Beta Scintillation Counter (Wallac, Finland). The percentage of specific lysis was calculated as 100 x [(experimental release – spontaneous release)/ (maximum release – spontaneous release)]. Spontaneous and maximum release were determined in the presence of medium or 1/6 N HCl, respectively.

Results

Relative Efficacy of ErbB-2 (E2) DNA vaccines

DNA vaccination was tested in six independent experiments (Table I). BALB/c mice were immunized three times at two week intervals with pCMV, pCMV-ErbB-2 (E2), pCMV-ErbB-2A (E2A), pCMV-cytoplasmic ErbB-2 (cytE2) or pCMV-cytoplasmic ErbB-2A (cytE2A). At two weeks after the last vaccination, mice were challenged s.c. with BALB/c mammary tumor D2F2 expressing human ErbB-2 (D2F2/E2). All mice injected with pCMV control vector developed tumors within 2 weeks. At six weeks after vaccination, only $8 \pm 7\%$ of mice vaccinated with E2 developed tumors, conferring >90% protection. Vaccination with E2A resulted in ~ 60% protection. CytE2 or cytE2A induced poor anti-tumor immunity protecting only 30 and 10% of immunized mice, respectively. These findings are consistent with our preliminary observation that transmembrane, but not cytoplasmic, ErbB-2 DNA vaccination resulted in significant protection against D2F2/E2. Several mechanisms, including antibody production, may contribute to differential anti-tumor immunity and this was analyzed.

Induction of anti-ErbB-2 antibody by ErbB-2 derivatives

Sera was collected two weeks after the third vaccination, diluted 1:20 and anti-ErbB-2 antibody was measured by its binding to the breast cancer cell line, SKBR3, using flow cytometry. Vaccination with pCMV/E2 induced anti-ErbB-2 IgG in all mice (Figure 1). E2A induced lower level of antibody in some but not all vaccinated mice. CytE2 or cytE2A did not induce antibody in any of the mice. The specificity of anti-ErbB-2 antibody was verified by its binding to D2F2/E2 but not control D2F2 cells (data not shown). Therefore, cytE2 and cytE2A, which were synthesized in the cytoplasm and degraded promptly by the proteosome, did not induce anti-ErbB-2 antibodies.

Because the amount of antibody induced by DNA vaccination was low, the differential antibody induction was verified in mice bearing tumors expressing individual mutant ErbB-2 proteins. Mice were injected s.c. with $2x10^5$ D2F2 tumor cells transfected with E2, E2A, cytE2 or cytE2A. Sera was collected at 4 weeks after tumor injection when the tumors reached ~ 5 mm in diameter (Figure 2). The growth of D2F2/E2 or D2F2/E2A, but not D2F2/cytE2 nor D2F2/cytE2A, induced anti-ErbB-2 antibodies, consistent with the finding with DNA vaccination. The amount of antibody induced by tumor growth was 20 fold or higher than that induced by DNA vaccination. The large number of growing tumor cells provided abundant antigens to stimulate antibody production. Still, antibody was not elicited by the growth of D2F2 tumor expressing cytoplasmic forms of ErbB-2.

To test if CD4 T cells were required for antibody production, mice were injected i.p. with anti-CD4 mAb GK1.5 at 6 days before tumor cell injection and continued every 3 days for four weeks until sera was collected. Anti-ErbB-2 antibodies were detected in untreated mice but not in mice depleted of CD4 T cells (Figure 3). Therefore, induction of anti-ErbB-2 antibody is a CD4 T cell dependent process.

The subclass of IgG production is determined by CD4 T cells. Th1 cells, characterized by the production of IFN-γ, induce B cell isotype switch and IgG2a production and Th2 cells induce IgG1 secretion (21). In E2 DNA vaccinated

mice, IgG2a was the predominant antibody, indicating Th1 activation (Figure 4A). D2F2/E2 tumor growth in naive mice induced primarily IgG1 indicating Th2 activation (Figure 4B).

Inhibition of tumor growth by vaccination with cytoplasmic ErbB-2 and cytokine DNA

The prompt degradation of cytoplasmic ErbB-2 or ErbB-2A was expected to generate a complete repertoire of antigenic peptides for CD8 T cell recognition (19). The poor anti-tumor activity of cytE2 and cytE2A DNA vaccination may reflect the lack of antibody or CD4 T cell help. CD4 help may be replaced, at least in part, by cytokine co-stimulation. To test if exogenous cytokine can provide the necessary co-stimulation during cytE2A DNA vaccination, mice were vaccinated with a combination of cytE2 and cytokine DNA.

Mice were immunized three times at two week intervals with pCMV/cytE2A and pEFBos/IL-2 or pEFBos/GM-CSF (Figure 5). Of the ten immunized mice, eight were protected from D2F2/E2 tumor growth whether they received the co-vaccination with IL-2 or GM-CSF DNA. Immunization with pCMV/cytE2A only protected one mouse, consistent with our earlier finding. None of the mice receiving pCMV, pEFBos/IL-2, or pEFBos/GM-CSF were protected. Anti-ErbB-2 antibody was not detected in any of the mice after DNA vaccination (not shown) and protection against tumor growth may be largely the result of CD8 T cell activation.

Next, the induction of cytotoxic T cells by cytE2A and GM-CSF DNA vaccination was examined. Mice were sacrificed at 6 weeks after tumor challenge and splenocytes were prepared and stimulated *in vitro*. In our experience BALB/c mammary tumor cells were very poor antigen presenting cells (APC) and generally caused death of co-cultured lymphocytes (not shown). This may be due, at least in part, to the expression of FasL on their surface (our unpublished results). To provide appropriate *in vitro* stimulation to CTL, APC were engineered. BALB/c 3T3 cells were transfected with wild type ErbB-2 and K^d. Cell clones with stable expression of both ErbB-2 and K^d were selected.

CTL activity was measured by the ^{51}Cr release assay after splenocytes were cultured with irradiated 3T3 stimulator cells for 5-7 days. Lysis of D2F2/E2 was observed at effector: target ratio of 10:1 or higher using CTL from mice which were immunized with pCMV/cytE2A and pEFBos/GM-CSF, and had rejected D2F2/E2 tumor challenge (Figure 6A). Control D2F2 cells were not lysed (Figure 6B). The mice which were similarly immunized but failed to reject tumor did not demonstrate CTL activity. Mice immunized with control pCMV, pCMV/cytE2A or pEFBos/GM-CSF developed tumor from the challenge and CTL was not detected. These results indicated the expansion of CTL in cytE2A and GM-CSF vaccinated mice following tumor rejection.

Cytoplasmic ErbB-2 may be more effective than the transmembrane ErbB-2 at producing MHC class I peptides. It may be advantageous to include cytoplasmic form of ErbB-2 in all vaccine regimens to enhance CD8 T cell activation. This hypothesis was tested by immunizing mice with 50 μg each of E2A and cytE2A (Figure 7). Control groups received 100 μg of E2A or cytE2A. All mice which received the combination vaccine, rejected tumor growth, whereas

50% and 20% of mice rejected tumor after they were immunized with E2A and cytE2A, respectively. The synergistic anti-tumor effect of co-vaccination with E2A and cytE2A is consistent with the notion that cytoplasmic ErbB-2 enhanced CTL activation.

Protection against tumors which do not express B cell epitopes

Vaccination with cytE2A and cytokine DNA did not elicit an antibody response but protected mice from D2F2/E2 tumor. It is possible that the tumor cells themselves may activate CD4 T cell and antibody responses which contribute to the observed tumor rejection. To test the anti-tumor activity of cytE2 vaccination without potential help from tumor associated transmembrane ErbB-2, D2F2 expressing cytoplasmic ErbB-2 (D2F2/cytE2) was used as the challenging tumor. Mice were immunized with pCMV/cytE2 and pEFBos/GM-CSF and challenged with D2F2/cytE2 (Table II). Control mice received pCMV, pCMV/E2, pCMV/cytE2 or pEFBos/GM-CSF. D2F2/cytE2 tumor was less tumorigenic than D2F2/E2 and half or less of the mice developed tumors when 2 x 10^5 tumor cells were injected (experiments 1-3). When 2.5 x 10^5 D2F2/cytE2 cells were injected, all mice developed tumors (experiment 4). Vaccination with E2 provided moderate to no protection against D2F2/cytE2. Vaccination with either cytE2 or GM-CSF DNA alone was not protective and cytE2 vaccination sometimes enhanced growth of D2F2/cytE2 tumor. Co-vaccination with cytE2 and GM-CSF effectively protected against D2F2/cytE2. Anti-ErbB-2 antibody was not detected in any of the mice at any time during the experiment (not shown). Therefore, antibody independent anti-tumor immunity was achieved by co-vaccination with DNA encoding cytoplasmic antigen and cytokine. These results demonstrated the feasibility and importance of segregating different immune effectors in anti-tumor immunity.

Discussion

Vaccination with E2, E2A, cytE2, or cytE2A resulted in approximately 90, 60, 30 and 10% protection against D2F2/E2 tumor, respectively (Table I). All recombinant proteins contained the entire ErbB-2 structural sequence, but the subcellular localization, membrane stability, and tyrosine kinase activity significantly affected their immunogenicity. Vaccination with transmembrane E2 DNA induced both cellular and humoral immunity and was strongly protective against D2F2/E2, but poorly effective against D2F2/cytE2 tumor (Table II). In this case, anti-ErbB-2 antibody may contribute to tumor rejection. Challenge with D2F2/E2 enhanced anti-ErbB-2 antibody production and tumor rejection. Anti-ErbB-2 antibody would not affect D2F2/cytE2 tumor growth nor did the tumor enhance antibody production. Co-vaccination with cytE2 and cytokine DNA, however, was highly effective against both D2F2/E2 and D2F2/cytE2 tumors. This was attributed to CTL activity which was detected in immunized mice after they rejected D2F2/E2 tumors (Figure 6). Without tumor challenge, CTL activity was low (not shown). These results indicated that the CTL population was amplified after the mice were exposed to and rejected antigen positive tumors. Enhanced presentation of CTL reactive peptides from cytE2 was further

supported by the observation that co-vaccination with E2A and cytE2A resulted in synergistic anti-tumor activity when compared to either vaccine alone.

Anti-ErbB-2 antibody induced by E2 or E2A DNA vaccination was primarily IgG2a, indicating the activation of Th1 cells. IgG1 was induced in tumor bearing mice, indicating a Th2 response (Figure 4). It is not clear if different antibody isotypes render different anti-tumor activity, although Th1 responses have been associated with anti-tumor effect (22). Anti-ErbB-2 antibodies may exert anti-tumor activity via classical pathways such as complement fixation and antibody dependent cell mediated cytotoxicity or by inducing apoptosis via truncated signaling (23). But antibodies have also been shown to interfere with anti-tumor immune T cell activity, implicating antibody production as a negative factor in anti-tumor activity (10;11). Induction of both humoral and cellular immune responses by ErbB-2 vaccination protected mice from D2F2/E2, but not D2F2/cytE2 tumor. This inadequacy may be due to weak CTL activity, an interference of CTL activity by some of the antibodies, or a combination of both. With a comprehensive immune response to ErbB-2 that activates all effector arms, it is not possible to dissociate the roles of each components. Here we have demonstrated the feasibility of inducing effective anti-tumor cellular immunity without anti-ErbB-2 antibody. If antibodies to a particular epitope prove to be safe and beneficial, it will be advantageous to elicit such antibody with defined ErbB-2 peptide fragments rather than whole protein (24).

Consistent with our earlier findings, ErbB-2 vaccines with lysine to alanine substitution at amino acid 753 in the intracellular domain (E2A and cytE2A) were less effective than their native counterparts. The single point mutation eliminated tyrosine kinase activity and correlated with decreased membrane stability of ErbB-2A. The expression level of E2A in transfected cells was about half that of E2 when measured by flow cytometry and Western blotting (not shown). It is possible that interaction between E2A and chaperon proteins, such as grp94 in the endoplasmic reticulum (25) and hsp90 in the cytoplasm (26), was altered by the mutation, resulting in reduced stability. The mutation may also alter the interaction between E2A and the ubiquitin ligase, c-Cbl, to accelerate E2A degradation (27). Any of these mechanisms may reduce stability and alter processing of E2A for T cell activation.

It is not clear how membrane associated ErbB-2 is processed through the MHC class II processing pathway for CD4 T cell activation. Transmembrane ErbB-2 shed from tumor cells may be phagocytosed and re-processed by antigen presenting cells (APC). When ErbB-2 is activated by heterodimerization with other members of the ErbB-2 family, the complexes are endocytosed and may be directed to the lysosome for degradation (28). In the lysosome, ErbB-2 may be degraded into peptides that can be presented with MHC class II molecules to ErbB-2 specific CD4 T cells. Because of its cytosolic localization and rapid degradation, cytoplasmic ErbB-2 will not be targeted to the lysosome and will not be a candidate for the MHC class II processing pathway and ,therefore, be unable to activate CD4 T cells.

Presentation of MHC class I peptides without co-stimulation signals may result in suppressed or anergized anti-tumor CTL. Vaccination with cytE2 alone

and challenge with D2F2/cytE2 tumor sometimes resulted in enhanced tumor growth (Table II), indicating a suppressed or anergized CD8 T cell response. Garza et al have shown in a LCMV gp transgenic system, immunization with LCMV gp peptide could induce activation and expansion of antigen specific CTL (29). In the absence of activated antigen presenting cells, these activated T cells were rapidly deleted and tolerance was induced. In the current study, vaccination with cytoplasmic ErbB-2 may be comparable to vaccination with the entire repertoire of MHC I associated peptides. Without co-stimulation signals, a short-lived CTL response may be induced. Only by co-vaccination with a cytokine gene was an effective anti-tumor response observed. Expression of IL-2 at the site of vaccination may provide signals for CTL survival and expansion. Co-expression of GM-CSF may recruit and activate APC to process and present ErbB-2 epitopes for full CTL activation.

Vaccination with cytoplasmic ErbB-2 alone may also induce the expansion of regulatory T cells which can down regulate the immune response. T cells have been shown to acquire MHC class I / peptide complexes from APC through endocytosis and become sensitized to killing by other activated CTL (30). If CTL acquired MHC class I / ErbB-2 peptide from APC without additional activation signals, these T cells may be lysed by other activated CTL, resulting in a shut-down of the immune response. Other regulatory T cells have been shown to acquire and present antigen to activated syngeneic CTL, and send death signals, thus preventing an antigen specific immune response (31). It remains to be determined if vaccination with cytoplasmic ErbB-2 activates any antigen-specific regulatory T cells capable of suppressing anti-ErbB-2 immune response. Cytoplasmic ErbB-2 may be a tool to turn on negative or regulatory T cell response when administered alone and to promote strong anti-ErbB-2 CTL response when administered with co-stimulation cytokines.

Results from this study demonstrated the feasibility of turning on a full panel of anti-tumor CTL without the involvement of antibodies. The critical role of co-stimulatory signals in CTL activation is emphasized. With this test system, the positive and negative effect of anti-ErbB-2 antibodies in tumor rejection can be defined without ambiguity. ErbB-2 based vaccination and immunotherapy can be designed rationally with these tools and knowledge. The same principles can be applied to improve the efficacy of most vaccines.

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Footnotes

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[3]Abbreviations used in this paper: E2, wild-type ErbB-2; E2A, tyrosine kinase deficient ErbB-2; cytE2, full-length ErbB-2 targeted to the cytoplasm; cytE2A, tyrosine kinase deficient cytE2.

Figure legends

Figure 1: Induction of anti-ErbB-2 antibodies by DNA vaccination. BALB/C mice (n=8) were immunized three times at two week intervals with 100 μg of wild-type or mutant ErbB-2 DNA as indicated. Serum was collected after the third DNA vaccination. Anti-ErbB-2 IgG antibody was measured by binding to SKBR3 cells and measured by flow cytometry. Whole anti-ErbB-2 IgG was detected. Each dot represents an individual mouse. The results are expressed as mean channel fluorescence. * indicates $p < 0.001$ by the Student's t test as compared to pCMV vaccinated mice.

Figure 2: Induction of anti-ErbB-2 antibodies after tumor growth. BALB/C mice (n=4) were injected with $2x10^5$ D2F2 tumor cells over-expressing wild-type or mutant ErbB-2 proteins. Sera were collected four weeks after tumor injection. Whole anti-ErbB-2 IgG was measured by flow cytometry. * indicates $p < 0.001$ by the Student's t test as compared to mice receiving D2F2 parental tumor.

Figure 3: Anti-ErbB-2 antibody production after CD4 T cell depletion. BALB/C mice (n=4) were untreated or depleted of CD4 T cells six days prior to injection of $2x10^5$ D2F2/E2 cells. Depleted state was maintained by i.p. injections of anti-CD4 antibody (GK1.5) every three days. Serum was collected four weeks after tumor challenge and incubated with SKBR3 cells. Anti-ErbB-2 IgG was measured by flow cytometry.

Figure 4: Anti-ErbB-2 IgG subsets induced by E2 vaccination or tumor growth. Serum was collected one week after the third pCMV/E2 DNA vaccination as described. IgG1 and IgG2a subtypes were measured (A). Serum was collected four weeks after injection of $2x10^5$ D2F2/E2 cells. IgG1 and IgG2a subsets wer measured (B). The results are expressed as mean channel fluorescence. The mean channel fluorescence for naïve mouse serum IgG1 was 6 and IgG2a was 7. ** indicates $p < 0.01$ and * indicates $p < 0.001$ by the Student's t test as compared to naïve mice.

Figure 5: Co-vaccination with cytE2A and cytokine DNA. Mice (n=10 per group) were vaccinated three times at two week intervals with 100 μg of the indicated plasmid DNA. Mice were challenged with $2x10^5$ D2F2/E2 cells and the percent of tumor free animals was recorded weekly for six consecutive weeks.

Figure 6: ErbB-2 specific CTL are present in tumor-free mice vaccinated with cytE2A and GM-CSF. BALB/C mice were vaccinated three times at two week intervals with pCMV, GM-CSF, cytE2A, or cytE2A + GM-CSF. Two weeks after final DNA vaccination, mice were challenged with $2x10^5$ D2F2/E2 tumor. At six weeks after tumor challenge, splenocytes were isolated and incubated for 7 days with irradiated 3T3/K^d/E2 stimulator cells and used in a 4 hour chromium-release assay. D2F2/E2 (A) and D2F2 (B) were used as target cells. * indicated p <

0.01 as compared to lysis by pCMV vaccinated mice. This experiment was repeated two times with similar results.

Figure 7: Co-vaccination with E2A and cytE2A results in tumor protection. BALB/C mice were vaccinated three times at two week intervals with 100 μg of pCMV, E2A, cytE2A, or 50 μg each of cytE2A and E2A. Two weeks after the third DNA vaccination, mice were challenged with $2x10^5$ D2F2/E2 cells. Tumor incidence was measured weekly.

Table I: *Induction of anti-tumor immunity with ErbB-2 DNA vaccines*

Vaccination[a]	D2F2/E2 tumor incidence[b] Expt 1	2	3	4	5	6	Total Tumor incidence[c] (mean ± s.d.)
pCMV	8/8	8/8	8/8	8/8	8/8	5/5	100 ± 0
E2	0/8[d]	1/8[d]	1/8[d]	-	-	-	8 ± 7
E2A	3/8[d]	4/8	-	4/8	3/8[d]	2/5	43 ± 7
cytE2	7/8	-	4/8	-	-	-	69 ± 27
cytE2A	8/8	-	-	8/8	6/8	4/5	89 ± 13

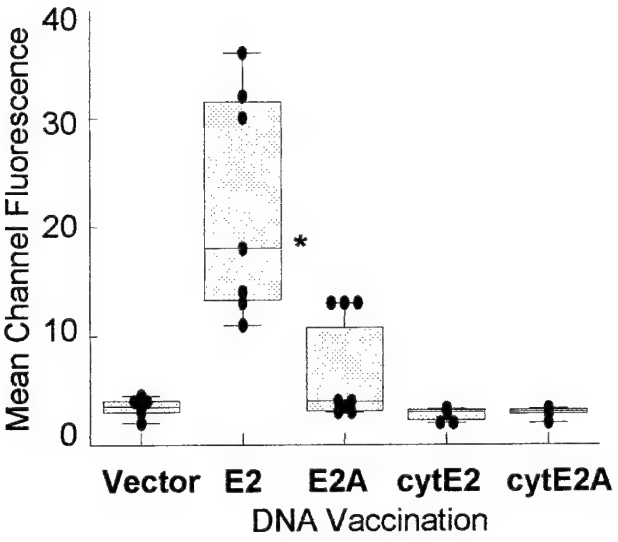
[a]Mice were vaccinated with DNA constructs as described in Materials and Methods. The results of six independent experiments are reported.
[b]Two weeks after the final DNA vaccination, mice were challenged s.c. with $2x10^5$ D2F2/E2 tumor cells. Tumor was palpated weekly and D2F2 tumor incidence is reported as total number of animals with palpable tumor at six weeks / total number of animals challenged with tumor.
[c]Total tumor incidence is the percentage of all animals from experiments 1-6 with palpable tumor.
[d] $p < 0.05$ by the Mantel-Haenszel Log-Rank Test as compared to pCMV vaccinated mice.

Table II: Anti-tumor immunity against D2F2/cytE2

	D2F2/cytE2 tumor incidence[b]				
Vaccination[a]	Expt	1	2	3	4[c]
PCMV		2/8	4/8	4/8	8/8
E2		2/8	1/8	-	7/8
cytE2		5/8	3/8	7/8	8/8
GM-CSF		4/8			8/8
cytE2 + GM-CSF		0/8			2/8[d]

[a]Eight mice in three independent experiments were vaccinated with DNA constructs as described in Materials and Methods.
[b]Two weeks after the final DNA vaccination, mice were challenged s.c. with $2x10^5$ D2F2/cytE2 tumor cells. Tumor was palpated weekly and tumor incidence is reported as total number of animals with palpable tumor / total number of animals challenged with tumor.
[c]These mice were vaccinated with cytE2A or cytE2A + GM-CSF and challenged s.c. with $2.5 x10^5$ D2F2/cytE2 tumor cells.
[d] $p < 0.05$ by the Mantel-Haenszel Log-Rank Test as compared to pCMV vaccinated mice.

Figure 1


Mean Channel Fluorescence
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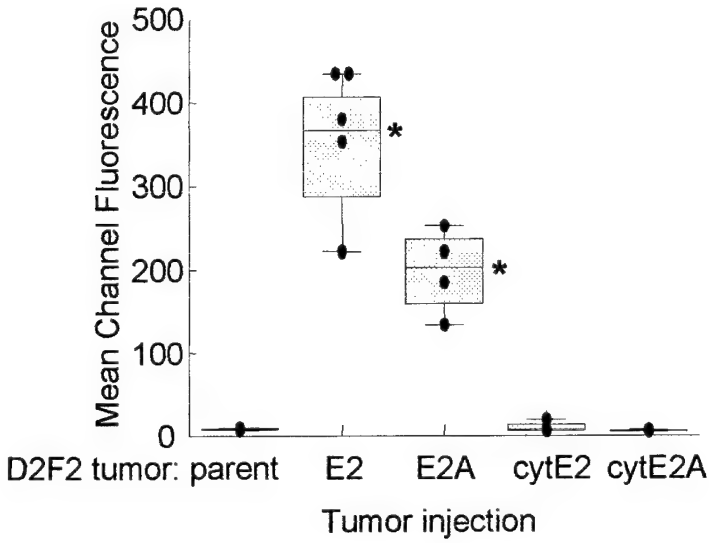
30
20
10
0
*
Vector
E2
E2A
cytE2
cytE2A
DNA Vaccination

Figure 2


Mean Channel Fluorescence
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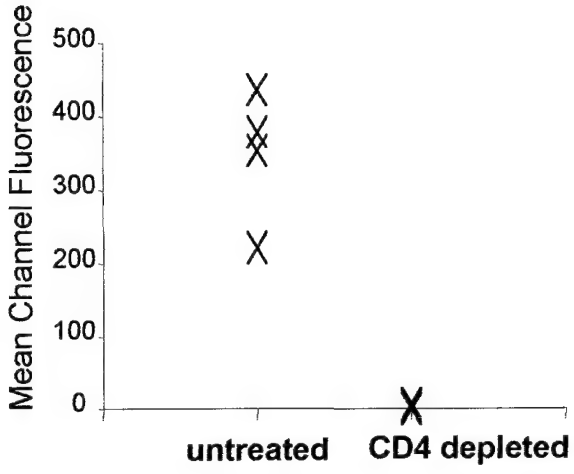
400
300
200
100
0
*
*
D2F2 tumor: parent
E2
E2A
cytE2
cytE2A
Tumor injection

Figure 3


Mean Channel Fluorescence
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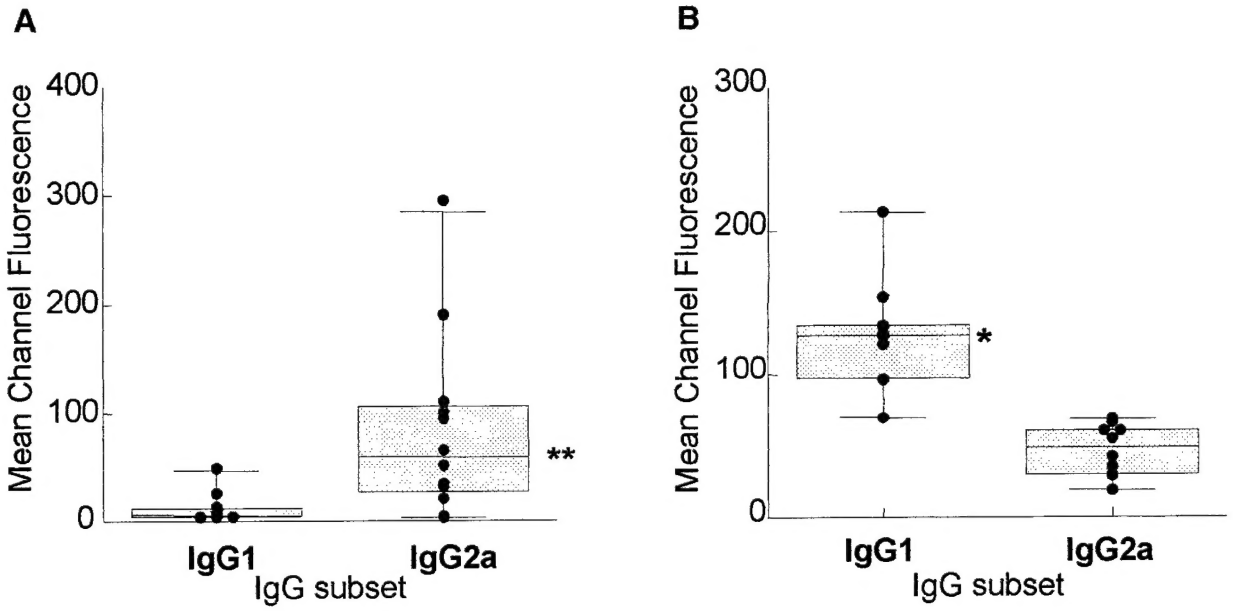
400
300
200
100
0
untreated
CD4 depleted

Figure 4


A
Mean Channel Fluorescence
400
300
200
100
0
**
IgG1
IgG2a
IgG subset
B
Mean Channel Fluorescence
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200
100
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*
IgG1
IgG2a
IgG subset

Figure 5

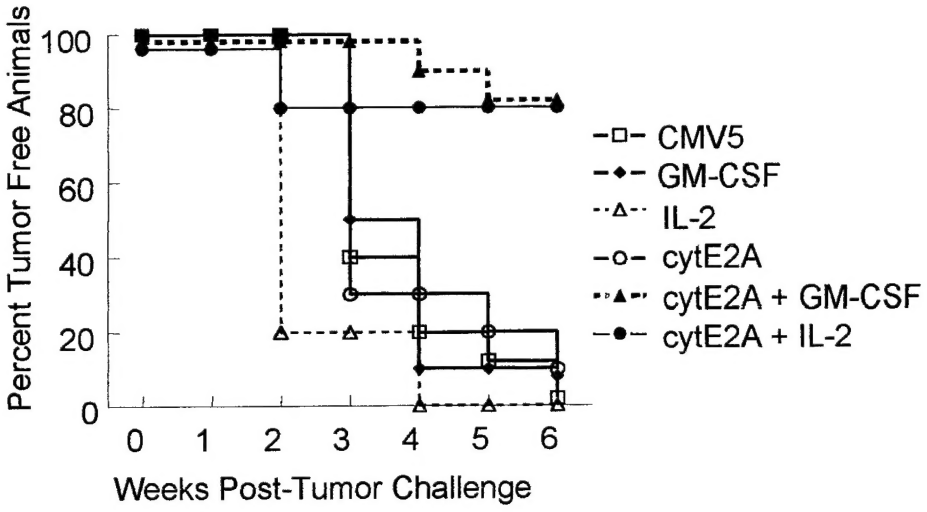

Percent Tumor Free Animals
100
80
60
40
20
0
0 1 2 3 4 5 6
Weeks Post-Tumor Challenge
CMV5
GM-CSF
IL-2
cytE2A
cytE2A + GM-CSF
cytE2A + IL-2

Figure 6

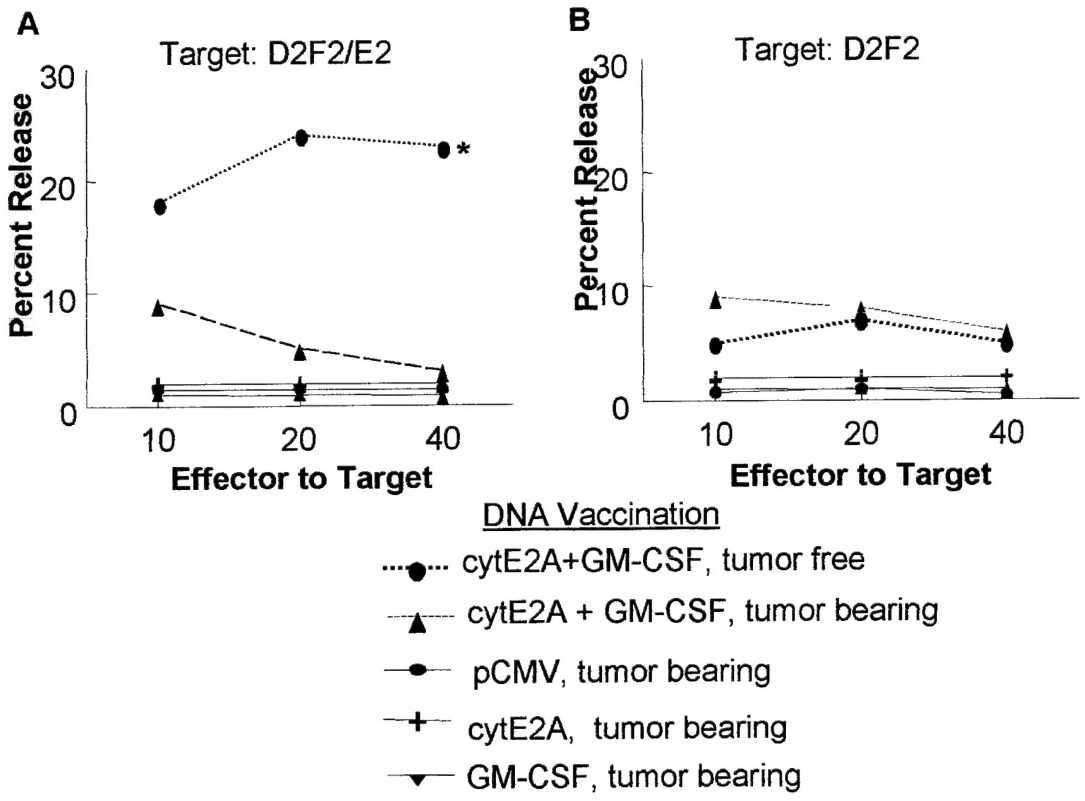
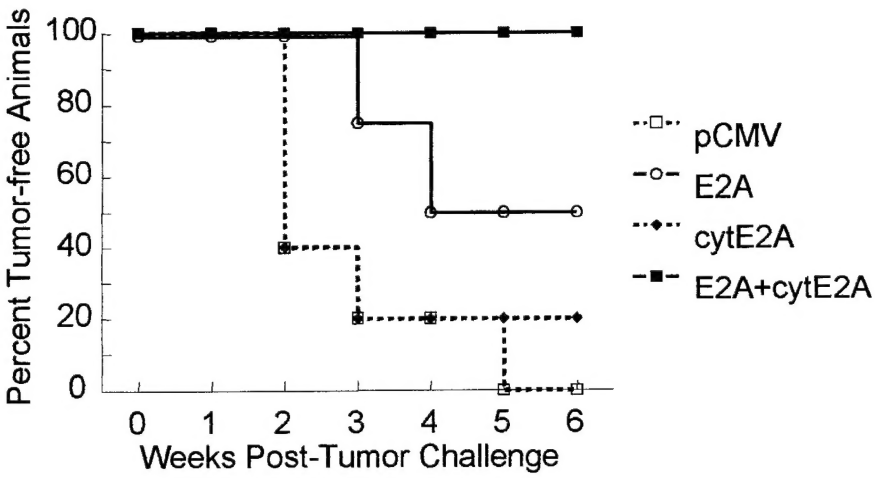

A
Target: D2F2/E2
Percent Release
30
20
10
0
10
20
40
Effector to Target
B
Target: D2F2
Percent Release
30
20
10
0
10
20
40
Effector to Target
DNA Vaccination
cytE2A+GM-CSF, tumor free
cytE2A + GM-CSF, tumor bearing
pCMV, tumor bearing
cytE2A, tumor bearing
GM-CSF, tumor bearing

Figure 7


Percent Tumor-free Animals
100
80
60
40
20
0
0 1 2 3 4 5 6
Weeks Post-Tumor Challenge
pCMV
E2A
cytE2A
E2A+cytE2A